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(54) Title: DETECTION AND SPECIATION OF CAMPYLOBACTER

(57) Abstract

Method for detecting *Campylobacter* by PCR detection of DNA sequence, highly conserved between species *lari*, *coli*, *jejuni* and *upsaliensis*. Speciation between these four is possible as the PCR product is differentially cleaved by restriction endonucleases.

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DETECTION AND SPECIATION OF CAMPYLOBACTER

This invention relates to the detection and speciation of *campylobacter* bacteria, for example in clinical, environmental and food samples. In particular, this invention relates to a method of detecting whether a sample contains *campylobacter* and to a method of differentiating between the main *campylobacter* species *jejuni*, *coli*, *upsaliensis* and *lari*.

*campylobacter* species are recognised as the most frequent cause of bacterial gastroenteritis in the United Kingdom and many other countries throughout the world. In the U.K. approximately 90% and 10% of case isolates are identified as *campylobacter jejuni* and *campylobacter coli* respectively, plus a small number of other species such as *campylobacter upsaliensis* and *lari*. The majority of the infections are sporadic the source of which remains largely unknown although the importance of several vehicles is now recognised.

There is a known desire to be able to detect and differentiate species of *campylobacter*. However, it is also known that present *campylobacter* enrichment culture techniques lack sensitivity, making detection difficult. *campylobacter jejuni* does not multiply in foodstuffs and low numbers may be present together with a high background of indigenous microflora. Also, surface viable counts of *campylobacter* can decrease rapidly and cells that are potentially culturable are often lost before samples reach a laboratory for analysis. Another factor making detection problematic is that antibiotics used in culture enrichment media may damage already weakened *campylobacter*.

There are currently available assays for detection of a variety of food and water-borne pathogens; *L.pneumophila*,

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*V. vulnificus*, *enteroinvasive E. coli*, *Shigella*; but no satisfactory method of detecting *campylobacter* or distinguishing between the four main *campylobacter* species is known.

A method of detecting *campylobacter* has been published by Giesendorf, B A J, et al in Applied and Environmental Microbiology, December 1992, pages 3804-3808. The method detects the species *jejuni*, *Coli* and *lari*, and produces similar results to conventional methods but in a reduced time. The method suffers from a number of drawbacks. It does not enable detection of the species *upsaliensis*. Further, the method employs polymerise chain reaction (PCR) techniques but nevertheless requires a short enrichment culture before the PCR can be employed. Further still, the primer used for the PCR does not have the precise homology with DNA sequences in the three *campylobacter* species that can be detected using the method.

Another method for detecting *campylobacter jejuni* and *campylobacter coli* is known from Wegmuller, B E et al, Applied Environmental Microbiology, vol. 59, part 7, 1993 pages 2161-2165. The described method detects only the species *jejuni* and *coli*.

In addition to the above-identified problems with detection and speciation of *campylobacter*, recent work on *campylobacter jejuni* suggests that in certain circumstances it enters a "non-culturable, viable form" when subjected to environmental stresses, such as pH or temperature extremes, increased oxygen tension or nutrient depletion. In this form, *campylobacter* infectivity is maintained but the organisms cannot be cultured. Thus there exists a need for the improvement of methods of detection of non-culturable forms of *campylobacter*.

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It is an object of this invention to provide a method of testing for the presence of campylobacter that enables more efficient detection and eliminates or mitigates the problems with existing techniques. It is a further object to provide a method of distinguishing the campylobacter species *jejuni*, *coli*, *upsaliensis* and *lari*.

Accordingly, in a first aspect the present invention provides a method of testing for the presence of campylobacter, e.g. in a clinical, environmental or food sample, comprising the steps of performing polymerise chain reaction (PCR) using primers adapted to amplify a region selected from (a) a sequence of at least 72 base pairs from sequence ID No. 1 and (b) a sequence having sufficient homology with (a) such that formation of PCR product is correlated with presence of campylobacter; and determining if any PCR product is formed.

It is preferred that sequence (b) has at least 75% homology with (a), preferably at least 90% homology and more preferably at least 95% homology. It is also preferred that the primers are at least 12 nucleotides in length, preferably between 19-22 nucleotides in length. In particularly preferred embodiments of the invention the primers consist of at least 12 contiguous nucleotides selected from (1) sequence ID NO:2 and sequence ID NO:3, (2) sequence ID NO:4 and sequence ID NO:5 and (3) sequences having sufficient homology with (1) or (2) such that formation of PCR product is correlated with presence of campylobacter

PCR has become a well known and established tool for DNA analysis. A single gene sequence can be marketed from a large amount of other DNA and amplified to provide a suitable quantity for analysis. The basis of todays PCR was first published in 1971 by Kleppe, E et al, J. Mol. Biol., 1971, 56, 341. Further significant details and improvements on the PCR

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method have been added by Saiki, R K et al, science, 1985, 230, 1350 and Mullis, K B, sci. am. 1990, 262, 36.

As will be appreciated by a person of skill in the art familiar with the PCR, it is important to operate at a temperature suitable to ensure that the primers used are specific for the sequence desired to be identified and amplified. To this end it is convenient to carry out the PCR reaction using the method of the invention at temperatures of at least 40°C preferably at least 45°C and in a particularly preferred embodiment at 48-52°C.

The 1.9 kilobase fragment identified in sequence ID 1 is an underlying feature of this invention and has been found to be highly conserved between campylobacter isolates. The method of the invention confers the advantage that PCR product will only be detected when a campylobacter strain is found in the sample tested. The method also confers the advantage that it will detect non-culturable viable forms of campylobacter as well as viable cells. Thus the method is effective where other methods have not been able to detect any campylobacter.

It is preferable to use a primer sequence that will only bind to one specific region of sequence ID NO:1 and which will not engage in formation of primer dimers and thus contaminate the PCR. Examples of preferred primers for use in the method of the invention are shown in the sequence ID numbers 2 and 3 and sequence ID numbers 4 and 5. These primers form further aspects of the invention.

In a second aspect the invention provides a method of distinguishing between campylobacter species *jejuni*, *coli*, *upsaliensis* and *lari* in a DNA containing sample by performing PCR utilizing primers capable of amplifying a selected campylobacter DNA sequence, said sequence having restriction

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endonuclease sites specifically associated with different *campylobacter* species and then testing for digestion of the PCR product by the specific restriction endonucleases.

Thus, *campylobacter* DNA that is differentially cleaved by restriction endonucleases is amplified, subject to digestion by the endonucleases and identified as from a particular species.

Sequence ID NO:1 was isolated from *Campylobacter jejuni* and is known to have a particular characteristic pattern of cleavage by restriction endonucleases. *Campylobacter coli*, *upsaliensis* and *lari* contain sequences corresponding to sequence ID NO:1 that have altered patterns of cleavage characteristic of each species.

In an embodiment of the second aspect there is provided a method of distinguishing between *campylobacter* species *jejuni*, *coli*, *upsaliensis* and *lari*, e.g. in a clinical, environmental or food sample containing *campylobacter*, comprising the steps of:-

performing polymerise chain reaction (PCR) on the sample using primers adapted to amplify a region of DNA sequence ID No.1 that includes nucleotides 124-196, or using primers adapted to amplify a DNA region corresponding thereto; and

testing the PCR product for digestion by restriction endonucleases *Alu* I, *Dra* I and *Dde* I.

The method of the second aspect is advantageous because it enables accurate speciation between the four clinically most significant species. In particular, when amplifying region 124-196 of SEQ DI NO:1, the PCR product from *campylobacter*

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*jejuni* is cleaved by all three restrictions endonucleases, whereas the PCR product from species *coli* is not cleaved by Dra I, the PCR product from species *upsaliensis* is only cleaved by Dde I and PCR product from *lari* is only cleaved by Alu I. It is a straightforward matter for a person skilled in the art to identify whether the PCR product is cleaved by one or more of the above endonucleases and thus the method enables simple speciation of *Campylobacter* into *jejuni*, *coli*, *upsaliensis* or *lari*.

The embodiments of the first aspect of the invention described above form embodiments of the second aspect of the invention also, provided that primers are selected so as to be adapted to amplify at least nucleotides 124-196 of sequence ID NO:1, or a *Campylobacter* sequence corresponding thereto.

In a preferred embodiment of the second aspect the primers consist of at least 12 contiguous nucleotides from sequence ID NO:s 4 and 5. Where the primers are sequences ID NO:s 4 and 5 the PCR product is 256bp and the respective products of cleavage by Alu I, Dra I and Dde I differentiate between *jejuni*, *coli*, *upsaliensis* and *lari*.

In a further embodiment of the invention, increased sensitivity and specificity for the detection of the presence of *Campylobacter* DNA, e.g. in food and liquid samples, is provided by the following additional methodologies:

1. A nested PCR has been developed, and is performed by an additional round of amplification using primer sequences international primer sequences ID4 and ID5. Two exemplary primer sequences are identified as Cru 0476 (SEQ ID NO:6) and Cru 0474 (SEQ ID NO:7). Following the second round of amplification, an amplicon of approximately 173pb is obtained in the presence of *Campylobacter* DNA. This DNA fragment

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retains the sequences for the restriction endonucleases Alu 1, Dde 1, and Dra 1, thus still enabling the speciation of the contaminating campylobacter.

2. Additional increased sensitivity and specificity is optionally achieved by southern transfer of the amplified PCR products obtained using oligonucleotide primers ID4 and ID5, followed by hybridisation with an internal probe (e.g. SEQ ID NO:8 probe sequence). The probe sequence spans the restriction sites for speciation of the contaminating campylobacter and therefore restriction digest analysis can be used in conjunction with the probe hybridisation to confer additional specificity. The probe can be labelled, for example with digoxigenin, or radiolabelled.

The extraction procedures for food and environmental samples preferably use an internal standard to enable qualitative estimation of extraction efficiency and the effects of non-specific inhibition. The PCR "MIMIC" (Clontech Laboratories, Palo Alto, California) is a form of competitive PCR in which a non-homologous neutral DNA fragment is engineered containing the same primer templates as the target DNA. The ampilmer produced from this construct is a fragment either smaller or larger than the target product. Known amounts of construct are added to the PCR reaction, and compete for the same primers, acting as an internal standard. Where a mimic is used, the mimic sequence is capable of being amplified by the same primers that amplify, under PCR conditions, the campylobacter sequence. The mimic, if cleaved by restriction endonucleases, does not form fragments that interfere with detection and/or speciation of campylobacter-the mimic is said to be "neutral".

It is preferred to carry out the PCR steps of the invention also using a mimic. In an example, mimic DNA is added to the

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sample and PCR is performed according to the invention. The PCR product is analyzed. If mimic DNA ha been amplified, this indicates that the PCR reaction has occurred properly. The product can then be tested for products that indicate presence of campylobacter. If no mimic DNA is amplified then this indicates PCR has not fully been carried out, or has been inhibited in some way.

It is further preferred to carry out PCR using mimic DNA of known and varying quantities. After amplification, the various results are compared and it is observed which of the results has comparable amounts of amplified mimic and (if present) campylobacter DNA. Thus, an estimate of the quantity of campylobacter DNA in the original sample is obtained.

The methods of the invention are further illustrated by the further embodiments of the invention described in the following Examples:-

Example 1

The PCR assay was developed by the following steps:

1> Identification of a highly conserved, species specific clone from a random library of *Campylobacter jejuni* insert fragments, cloned in the vector pBlueScript KS.

2> Chain termination sequencing of the 1.9 kilobase fragment in both directions.

3> Selection of presumptive primer pairs based on regions of equivalent G+C/A+T content, and low identity (prevention of 'primer-dimer').

4> Optimisation of reaction parameters: Mg<sup>++</sup> concentration, Taq enzyme source, buffer composition, annealing temperature,

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cycling parameters.

Example 2

**ASSESSMENT OF ASSAY SENSITIVITY AND SPECIFICITY**

Using a single amplification (35 cycles, annealing temperature 50°C) we detected approx. 10 CFU/ml of *Campylobacter jejuni*.

We at this stringency, the assay was specific for *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter upsaliensis*. Using a lower annealing temperature (42°C), *Campylobacter fetus* and *Campylobacter lari* were also amplified.

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Example 3

The following procedures were used for PCR amplification of *Campylobacter jejuni* from milk and water samples.

- 1> cell lysis by boiling or freeze/thaw cycles, centrifuge, pcr supernatant directly.
- 2> Cell lysis by boiling, nucleic acid purification by phenol\chloroform extraction
- 3> cell lysis by guanidine isothiocyanate, nucleic acid purification using nuclease binding matrix ("isoquick").
- 4> Cell concentration using magnetic particles coated with anti-campylobacter igg, cell lysis by boiling.
- 5> concentration and immobilisation of cells on 0.2µm nitrocellulose filters ('solid-phase' pcr).
- 6> Cell concentration using affinity column purification
- 7> guanidium isothiocyanate nucleic acid extraction, with purification using silica bead matrix ('boom method')

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**EXTRACTION OF MILK SAMPLES**

**FOR PCR ANALYSIS**

WARM MILK TO 37°C

CENTRIFUGE @ 3,000xg, 15 MINUTES

CHILL ON ICE. SEPARATE MILK AND CREAM

CREAM

EMULSIFY IN 10 VOLUMES  
OF WARM PBS

CENTRIFUGE @ 9,000xg, 15 MINUTES

DISCARD SUPERNATANT

MILK



DISCARD SUPERNATANT

RESUSPEND MILK AND CREAM PELLETS IN  
5 VOLUMES OF PBS. POOL EXTRACTS.

BOIL FOR 10 MINUTES

CENTRIFUGE @ 14,000xg, 5 MINUTES

EXTRACT DNA WITH SILICA-BASED PURIFICATION MATRIX

ELUTE NUCLEIC ACIDS WITH 2x50µl PURE WATER

PCR NEAT SAMPLE, AND 10-FOLD SERIAL DILUTIONS

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### EXTRACTION OF WATER SAMPLES

PRE-FILTRATION THROUGH 30 $\mu$ m WHATMAN FILTER

↓  
 CENTRIFUGE @ 9,000 $\times$ g, 15 MINUTES  
 ↓  
 WASH PELLET x2, 1ML PBS  
 ↓  
 RESUSPEND IN 1ML STERILE WATER  
 ↓  
 BOIL, 10 MINUTES  
 ↓  
 EXTRACT DNA WITH SILICA-BASED  
 PURIFICATION MATRIX

Example 4

We observed the following differentiation of *Campylobacter* species using PCR primers SEQ ID NO:4 and SEQ ID NO:5 and restriction endonucleases Alu I, Dra I and Dde I.

Species	PCR product digested with:		
	Alu I	Dra I	Dde I
<i>C. jejuni</i>	+	+	+
<i>C. coli</i>	+	-	+
<i>C. upsaliensis</i>	-	-	+
<i>C. lari</i>	+	-	-

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We further observed the following fragment sizes for different species.

Species	Restriction enzyme digests of PCR amplicers				
	Alu I		Dde I		Dra I
Thermophilic/ enteropathogenic		Fragment sizes (bp)		Fragment sizes (bp)	Fragment sizes (bp)
C. jejuni	2	108, 148	2	83, 173	2
C. jejuni (hippurate + ve)	2	108, 148	2	83, 173	2
C. coli	2	108, 148	2	83, 173	1
C. lari	2	108, 148	1	256	1
C. upsaliensis	1	256	3	30, 83, 143	1

The results are also illustrated in Fig. 3 where bonds were not visible by eye they were detected by use of radiolabels.

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Example 5

To test the specificity of campylobacter detection we used PCR primers on laboratory samples containing a wide range of organisms. The primers were SEQ ID NO:s 4 and 5, PCR product size in brackets:

Species	Annealing temperature of primers		
	37°C	42°C	50°C
<i>C. jejuni</i> (256)	+	+	+
<i>C. coli</i>	+	+	+
<i>C. upsaliensis</i>	+	+	+
<i>C. fetus</i>	+	±	-
<i>C. lari</i>	+	±	-
<i>C. mucosalis</i>	±	-	-
<i>C. sputorum</i>	±	+	-
<i>Achromobacter sp.</i>	+	-	-
<i>Acinetobacter calcoac.</i>	± (multiple)	-	-
<i>Acinetobacter sp.</i>	± (multiple)	-	-
<i>Aeromonas hydrophila</i>	-	-	-
<i>Citrobacter freundii</i>	-	-	-
<i>Enterobact. aerogenes</i>	-	-	-
<i>Enterobact. agglomerans</i> (500)	±	-	-
<i>Enterobacter cloacae</i>	-	-	-
<i>Escherichia coli</i>	-	-	-
<i>Flavobacterium</i>	-	-	-

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Species	37°C	42°C	50°C
<i>Klebsiella aerogenes</i>	-	-	-
<i>Klebsiella oxytoca</i>	± (500)	-	-
<i>Proteus mirabilis</i>	-	-	-
<i>Proteus morganii</i>	-	-	-
<i>Providencia stuartii</i>	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-
<i>Pseudomonas maltophilia</i>	-	-	-
<i>Pseudomonas pickettii</i>	-	-	-
<i>Salmonella enteritidis</i>	-	-	-
<i>Salmonella typhimurium</i>	-	-	-
<i>Serratia marcescens</i>	-	-	-
<i>Serratia liquefaciens</i>	-	-	-
<i>Shigella dysenteriae</i>	-	-	-
<i>Shigella sonnei</i>	-	-	-
<i>Vibrio cholera</i>	-	-	-
<i>Vibrio furnassii</i>	± (1000)	-	-
<i>Vibrio parahaemolyticus</i>	± (180)	±	-
<i>Yersinia enterocolitica</i>	-	-	-
<i>Oxford staphylococcus</i>	± (300)	±	-

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Example 6

Using standard culture techniques (published by Bolton F J, et al, J. Appl. Bacteriol., 1983, vol. 54, pages 115-125) we compared the detection of *campylobacter jejuni* by culture with detection by the method of the invention (using primers SEQ ID NO:s 4 and 5) against time.

The success of culture detection declined over the time of the comparison, no culturable organisms being found remaining in the sample after 26 days - thus at this point detection by culture indicated no campylobacter present.

By contrast, using the PCR method of the invention we were still able to detect campylobacter DNA in a sample 42 days old. The results are illustrated in Figure 1.

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Example 7

To confirm the accuracy of the PCR method of the invention we tested many samples that contained known species of campylobacter. The results, illustrated in figs. 4-11, confirm the method is completely accurate for all samples tested, and correctly identified each one by species.

**FEATURES OF THE PCR ASSAY FOR *Campylobacter jejuni***

It allows rapid and sensitive detection of *Campylobacter jejuni* from environmental samples,

provides a semi-quantitative indication of the bacterial load, and determines whether samples are contaminated with *Campylobacter jejuni*, *coli*, *upsaliensis* or *lari*.

The method is of use for examining epidemiology of campylobacter infection such as a) seasonal peak, b) inverse correlation of surface water viable counts with human disease, c) role of water supply in (re)infection of broiler flocks with *Campylobacter jejuni*, d) contamination of foodstuffs at the point of sale, and e) determine origin of sporadic human infections.

Thus, a novel method incorporating polymerise chain reaction assay has been developed for the detection of campylobacter in clinical, environmental and food samples, such as milk and water samples. The assay is rapid, highly sensitive, and specific for *Campylobacter* sp. Simple restriction analysis of the PCR product allows speciation between *Campylobacter jejuni*, *coli* *upsaliensis* and *lari*.

Description of Drawings

Fig. 1 shows a comparison of culturability of *campylobacter jejuni* against time with detection of *campylobacter jejuni* using PCR of the invention;

Fig. 2 shows the sequence of open reading frame "C" from insert fragment pBSKSCJ19B with primer/nested primer locations, and restriction sites;

Fig. 3 shows restriction enzyme analysis of PCR products amplified from *C. jejuni*, *coli* and *upsaliensis*.

Fig. 4-11 shows the results of carrying out the PCR method of the invention on samples containing a wide ranges of known isolates. "P"=Penner Serotype Reference strains. "L"=Lior Serotype Reference Strains. Others are laboratory isolates. Standard size markers are on the gel ends.

Fig. 4      *C. Jejuni.*                  Alu I digest.

Fig. 5      *C. Jejuni.*                  Dde I digest.

Fig. 6      *C. Jejuni.*                  Dra I digest.

Fig. 7      *C. upsaliensis.*            Alu I and Dde I digest.

Fig. 8      *C. upsaliensis.*            Dra I digest.

Fig. 9      *C. Coli.*                    Alu I digest

Fig. 10     *C. Coli.*                    Dde I digest

Fig. 11.     *C. Coli.*                    Dra I digest

Sequence ID No. 1

1 accaacagcc attaaaaatc ttgactcagc catactcact ttaagaacac  
     tggttgtcgg taatttttag aactgagtgc gtatgagtga aattcttgc  
 51 gcgacatat ataataccgt tgccaaatc cctgaaagca taaaacccaa  
     cgccctggata tattatggca acgggttag ggactttcg atttgggtt  
 101 aatcacacact gaagtatgaa gtggtctaag tcttgaaaaa gtggcatatt  
     ttagtgtgga cttcatactt caccagattc agaactttt caccgtataa  
 151 gtcctggtaa ataatttaaa ttaggatatg ccattgaaa agctataaga  
     caggaccatt tattaaattt aatcctatac ggttaaacttt tcgatattct  
 201 gttcctatacg ccataccaac aatgccaaac aatatggtcg caaacataaa  
     caaggatatc ggtatggttt ttacgggtt ttataccagc gtttgtattt  
 251 atatcttgca accgtatagt cgtaatttaa tacattacct ggatgcattcg  
     tatagaacgt tggcatatca gcattaaattt atgtaatgga cttacgtac  
 301 actttctcct taaaattttt gataacaaga gaagattata gaatattaat  
     tgaaagagga attttaaaaa ctattgttct cttctaataat cttataatta  
 351 tatacatttt ttcttaaaaaa tgat/aatttt gttaatcatt tgttatgttt  
     atatgtaaaaa aagaattttt acta ttaaaa caatttagtaa acaatacaaa  
 401 tatattttaa ggctaaatca gtcttattta ttgatattta tcttataacc  
     atataaaattt ccgatattgt cagaataaaat aactataaaat agaatattgg  
 451 taaaccttgc acattttttt taaaatcttc acccacttta tctcttactc  
     atttgaacag tgtaaaaaat attttagaag tgggtgaaat agagaatgag  
 501 tttttataaa agttcta{aca gcagtatcgc tcacatgt}c acctatccaa  
     aaaaatattt tcaagat tgt cgtcatagcg agtgtaca g tggataggtt  
 551 acatttttct taatatcttc atgaaaaacc aaagctccag gttgctttaa  
     tgtaaaaaaga attatagaag tacgtttgg ttgcagggtc caacgaaattt  
 601 aagcaaagaa ataaaagcca attctttttt agttaaaaaca atttctccac  
     ttcgtttctt tattttcggt taagaaaaaa tcaattttgt taaagaggtg  
 651 cactgtaaat taaagttcgt ttattttgt taaattgata ttcttcagaa  
     gtgacattta atttcaagca aataaaaaca atttaactat aagaagtctt  
 701 atttttacaa gcatatttgc ttcaattttt tcacctatca gataatctaa  
     taaaaaatgtt cgtataaaacg aagttaaaaaa agtggatagt ctattagatt  
 751 aactttaaaac aactcttcta tatcaacagg tttaatcaaa tatttatactt  
     ttgaaatttg ttgagaagat atagttgtcc aaatttagttt ataaatagat

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Sequence ID No. 1 (cont)

801 taccaatatac aatagaacgc aaaagtctct ctt tctctga atacgcacta  
atggttatag ttatcttgcg tttcagaga gaa{agagact tatcggtatgat

851 aga acaacaa ttgggacatc atctgaaatt tcttaatct ctctgccat  
tct tggtgaa aaccctgttag tagactttaa agaaattaga gagaacggta

901 atccagtc当地 tccataatag gcatacaat atctgtgata actaaatctg  
taggtcaggtaggtt aggtattatc cgatcgat tagacactat tgatttagac

951 gcttaaattt tttaaatttt ttaagccccat catctccatt ttgagctccg  
cgaatttaaaa aaatttaaaa aattcggggt gtagaggtaa aactcgaggc

1001 attactttac taaagcgttc gcttaatata ttaatcattt attctctagc  
taatgaaatg atttcgcaag cgaatttat aatttagtaac taagagatcg

1051 cttaaccta tcttcaacta ctaatattat taattcttta cattcttgta  
gaattggagt agaagttgtt gattataata attaagaaat gtaagaacac

1101 acat/ttctac tctaccctct cttttagttt taaaaatatc tcaaaacaag  
tgta aagatg agatggaga gaaaatcaaa attttatag agttttgttc

1151 ccccgctttt tccatttttta acttttattt ttcccttggaa actttcgata  
ggggcagaaaa aggtaaaaat tgaaaataaa aaggaacctt tgaaagctat

1201 atttgc当地 ttatataaaag tcctactcct ataccttgac taggatgtt  
taaacagatg aatataatttc aggatgagga tatggactg atcctacaaa

1251 tggtaaaaa taaggttggaa aaattttatc taaattttct ttatcaatcc  
acaacattttt attccaactt tttaaaaat tagttaaaaaga aatagtttagg

1301 caccagcatt atctttattt gtaattttca gataattttt tccaaatttt  
gtggcgttaa tagaaaataa cattaaaatg ctattaaaaa agtttaaaa

1351 gaaaaattta ttgttatgat ttccctttt ttgttttaa atgcttctat  
cttttaaat aacaatacta aaaggaaaaa aacaaaaatt tacgaagata

1401 tgaatttaaa atcaaattaa gaaaaactct tattaaacca ttctcatatg  
actttaaattt tagtttaattt cttttgaga ataattttgtt aagagtatac

1451 caaaaacttc ataatcacctt ttccaaacaa tattaaatatt tacatgattt  
gttttgaag tattatgaa aagctttgtt ataattataa atgtactaaaa

1501 ttctatag ttccaaagc aatttccaag gctttattta aagtctcttt  
aaaagatatc aaagtttgc tttaagggttc cgaaataat ttccagagaaaa

1551 tataaataca cactgctcta ctcccttggaa aacaaaatgtt ctaaacacat

1601 caattgttgc tgacatattt ttaatcatat ctttgattt tgagtaaattt  
gttaacaaag actgtataaa aatttagtata gaaaactaac actcatttaa

1651 tcagcaaatc cttttcatc tttaagattt tgcttcattt gaaacatggc  
agacgtttag gaaaaatgtt aattctaaa acgaagtaaa ctggatccg

- 21 -

Sequence ID No. 1 (cont)

1701 aataccgagc tcatttaacg gttgtctcca ttgatgtgct atatcactaa  
ttatggctcg agtaaattgc caacagaggt aactacacga tatagtgatt

1751 tcatttggtc taatgaagat ttcaaaatct cttcatatgc tattttaaa  
agtaaacaag attacttcta aagttttaga gaagtatacg ataaaattat

1801 tcttttcat tttttccaa ggcaatttgc attttttct caaattttt  
agaaaaagta aaaaaagggtt ccgttaaacg taaaaaaaaa gttaaaaaaaa

1851 acctaactgt ataaattctt gttggtgatt tttaactgta ttttcaagat  
tggattgaca tatttaagaa caaccactaa aaattgacat aaaagaacta

1901 taatacttaa ttctcttaat tttagcgtgat tttagagcaag ctcttcat  
attatgaatt aagagaatta aatcgcacta aatctcggtc gagaagta

- 22 -

**Sequence ID No. 2**

5' TCTTAGTGCG TATTCAGAGA 3'

**Sequence ID No. 3**

5' ACAGCAGTAT CGCTCACATG T 3'

**Sequence ID No. 4**

5' AGAACACGCG GACCTATATA 3'

(also referred to as B04263)

**Sequence ID No. 5**

5' CGATGCATCC AGGTAATGTA T 3'

(also referred to as B04264)

Primer SequencesInternalCru 0476 (SEQ ID NO: 6)

5' a t c a c a c c t g a a g t a t g a 3' 18 mer

Cru 0474 (SEQ ID NO: 7)

5' t a c g a c t a t a c g g t t g c a 3' 18 mer

Amplimer size: 173 base pairs

- 24 -

SEQ ID NO: 8

Probe sequence (173 bp amplimer from nested primers)

tacgactatacggttgcaagatatttatgtttgcg  
 -----+-----+-----+-----+  
 atgctgatatgccaacgttctataaaatacaaacgc

Y D Y T V A R Y F M F A

accatattgttggcattgttgtatggct  
 -----+-----+-----+1740  
 tggtataacaaaccgttaacaaccataccga  
 T I L F G I V G M A -

Alu I  
 1741 ataggaactcttagctttcaaattggca  
 -----+-----+-----+  
 tatccttgagaatatcgaaaagttaccgt  
 I G T L I A F Q M A -

Dra I  
 tatcctaatttaattttaccaggacaa  
 -----+-----+-----+1800  
 ataggattaaatttaataatggtcctgtt  
 Y P N L N Y L P G Q -

Dde I  
 1801 tatgccacttttcaagacttagaccacttcatacttcagggtgtat  
 -----+-----+-----+-----+  
 atacggtaaaaagttctgaatctggtaagtatgaagtccacacta  
 Y A T F S R L R P L H T S G V I

- 25 -

PCR MIMIC primers and sequence

Primer 1 Cru 0477 (SEQ ID NO: 9)

5' agaacacgcggaccatatatacgcaagtgaaatctcctccg 3' 40 mer

Primer 2 Cru 0660 (SEQ ID NO: 10)

5' cgatgcattccaggtaatgtattctgtcaatgcagttttag 3' 41mer

MIMIC SEQUENCE (SEQ ID NO: 11)

5' agaacacgcg gacctatata cgcaagtgaa atctcctccg  
tcttggagaa gggagagcgt ttgccccagc taccattgat  
gtgtacatga tcatttgtcaa atgctggatg attgatgcag  
acagccgtcc caagttcgt gagctgattg cagagttctc  
caaaatggct cgtgaccctc cccgctatct tgttatacag  
ggagatgaaa ggatgcactt gcctagccct acagattcca  
agtttatcg caccctgatg gaggaggagg acatggaaga  
cattgtggat gcagatgagt atcttgccttcc acaccaggc  
tttttcaaca tgccctctac atctcggact cctcttctga  
gttcattgag cgctactagc aacaattctg ctacaaactg  
cattgacaga 3'

CLAIMS:

1. A method of testing for the presence of campylobacter in a clinical, environmental or food sample comprising the steps of:-

performing polymerise chain reaction (PCR) using primers adapted to amplify a region selected from (a) a sequence of at least 72 base pairs from sequence ID No. 1 and (b) a sequence having sufficient homology with (a) such that formation of PCR product is correlated with presence of campylobacter; and determining if any PCR product is formed.

2. A method according to claim 1 in which sequence (b) has at least 75% homology with (a), preferably at least 90% homology and more preferably at least 95% homology.

3. A method according to Claim 1 or 2 in which the primers comprise at least 12 nucleotides.

4. A method according to Claim 3 in which the primers are 19-22 nucleotides in length.

5. A method according to Claim 3 or Claim 4 in which the primers consist of at least 12 nucleotides selected from:

- (1) sequence ID No.2 and sequence ID No.3,
- (2) sequence ID No.4 and sequence ID No.5, and
- (3) sequences having sufficient homology with (1) or (2) such that formation of PCR product is correlated with presence of campylobacter.

6. A method according to any previous claim in which the temperature of the PCR is sufficiently high to prevent the primers annealing with non-campylobacter DNA.

7. A method according to Claim 6 in which the PCR temperature is at least 40°C.

8. A method according to Claim 7 in which the PCR temperature is at least 45°C.

9. A method according to Claim 8 in which the PCR temperature is 48-52°C.

10. A method of distinguishing between *campylobacter* species *jejuni*, *coli*, *upsaliensis* and *lari* in a DNA containing sample by performing PCR utilizing primers capable of amplifying a selected *campylobacter* DNA sequence, said sequence having restriction endonuclease sites specifically associated with the different *campylobacter* species, obtaining PCR product and then testing for digestion of the PCR product by the specific restriction endonucleases.

11. A method according to claim 10 wherein the selected *campylobacter* sequence is differentially cleaved by the restriction endonucleases Alu I, Dra I and Dde I

12. A method of distinguishing between *campylobacter* species *jejuni*, *coli* and *upsaliensis* comprising the steps of:-

performing polymerise chain reaction (PCR) using primers adapted to amplify a region of DNA sequence ID No.1 that includes nucleotides 124-196, or using primers adapted to amplify a region of *campylobacter* DNA corresponding thereto; and

testing the PCR product for digestion by restriction endonucleases Alu I, Dra I and Dde I.

13. A method according to Claim 10, 11 or 12 in which *campylobacter jejuni* is characterised by cleavage by all three endonucleases, *coli* is characterised by loss of cleavage by Dra I endonucleases, *upsaliensis* is characterised by loss of cleavage by Alu I and Dra I endonucleases and *lari* is characterised by cleavage by Alu I only.

14. A method according to any of Claims 10 - 13 characterised by the features of any of claims 3-9.

15. Use of restriction endonucleases Alu I, Dra I and Dde I in differentiating between *campylobacter* species *jejuni*, *coli* *upsaliensis* and *lari*.

16. A PCR primer having the sequence of sequence ID No. 2.

17. A PCR primer having the sequence of sequence ID No. 3.

18. A PCR primer having the sequence of sequence ID No. 4.

19. A PCR primer having the sequence of sequence ID No. 5.

20. A PCR primer having the sequence of sequence ID No. 6, 7, 9 or 10.

21. A kit for detecting *campylobacter* comprising one or more reagents for carrying out the method of any of claims 1-9.

22. A kit for determining *campylobacter* species comprising one or more reagents for carrying out the method of any of Claims 10-15.

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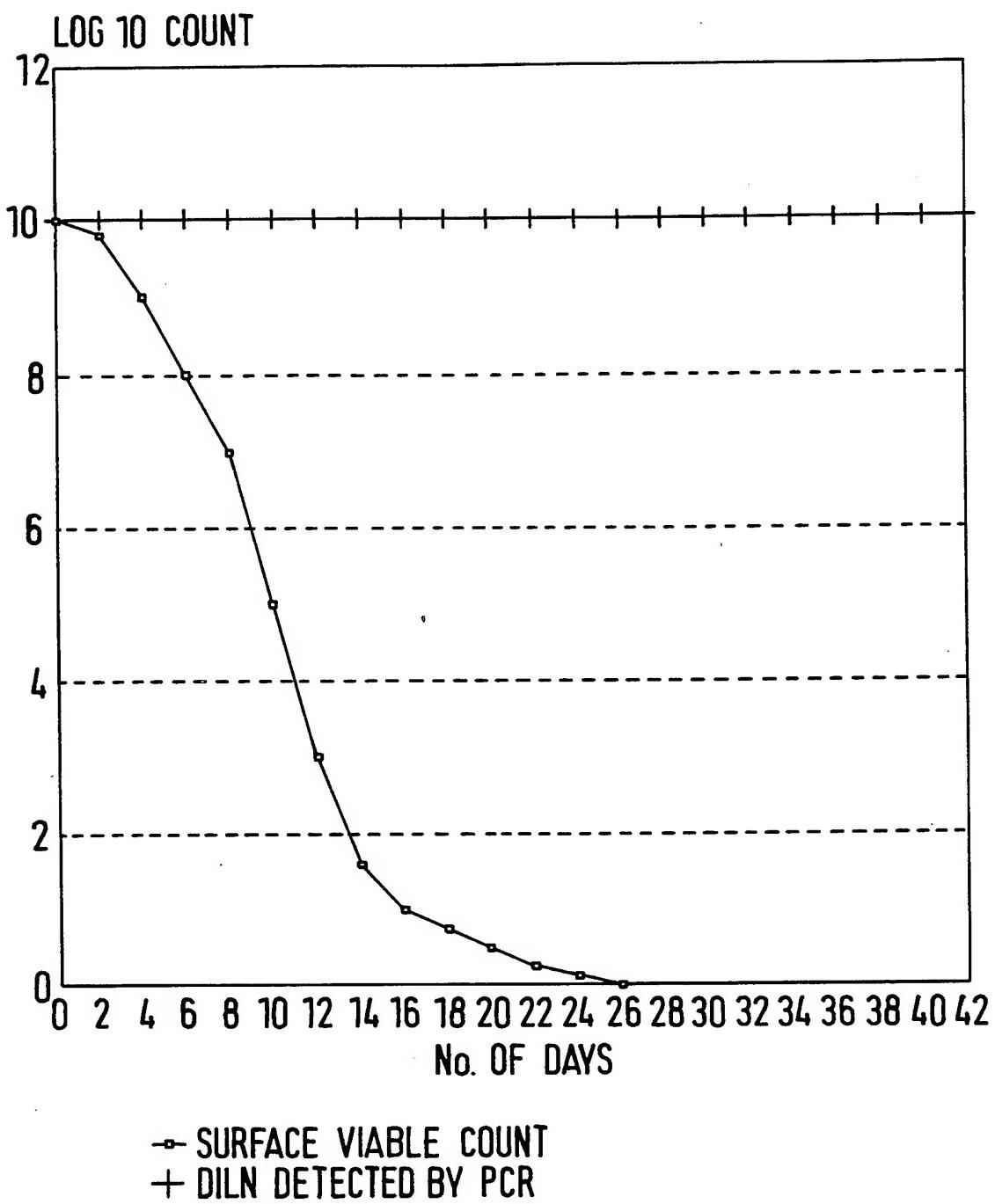
CULTURABILITY AND PCR DETECTION OF  
*C. JEJUNI* IN A POND WATER MICROCOsm

FIG. 1

tgattaaacaaaatttatcatttttaagaaaaatgtataatttataatcttc  
- - - - + - - - + - - - + - - - + - - - + - - - +  
acttaatttgttttaataqtaaaaattttacatataattaaatggatatttagaaag

D \* Q N Y H F \* E K M Y N \*  
\* L T K L S F L R K N V \* L I F Y N L L -

FIG. 2(I)

tttgttatcaaaaatttaaggagaaggatccaggatcgatccaggtaatgtattaaattacgac  
- - - - + - - - + - - - + - - - + - - - + - - - + - - - + - - - +  
aacaatagttttaaaattccctttagctacgttaggtccattacataatttaaatggctg

L L S K I L R K S M H P G N V L N Y D -

**tat** tacggtttgcagaagataattttatgttttgccatatttgtggcatatgttttggttatggct  
ataatgccaacgttctataaaaataccaaacqctggataacaaccataccga

Y T V A R Y F M F A T I L I V G M A -

Alu I Dra I

ataggaactcttatacgctttcaaatggcataatccataattttaccaggacaa  
- - - + - - - + - - - + - - - + - - - + - - - + - - - +  
tatcccttgagaatatcgaaaagtttacccgtataggattaaatttaaatggatccgttt

I G T L I A F Q M A Y P N L N Y L P G Q -

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Dde I

tatgccactttcaaggacttagaccacttcatacttcagggtgtgattttgggttttatg  
 -+-----+-----+-----+-----+-----+-----+  
 atacggtgtaaaaagttctgtgaatctggtgtgaagtatgtgaaggtaatccacactaaaaacaaatac  
 Y A T F S R L R P L H T S G V I F G F M -

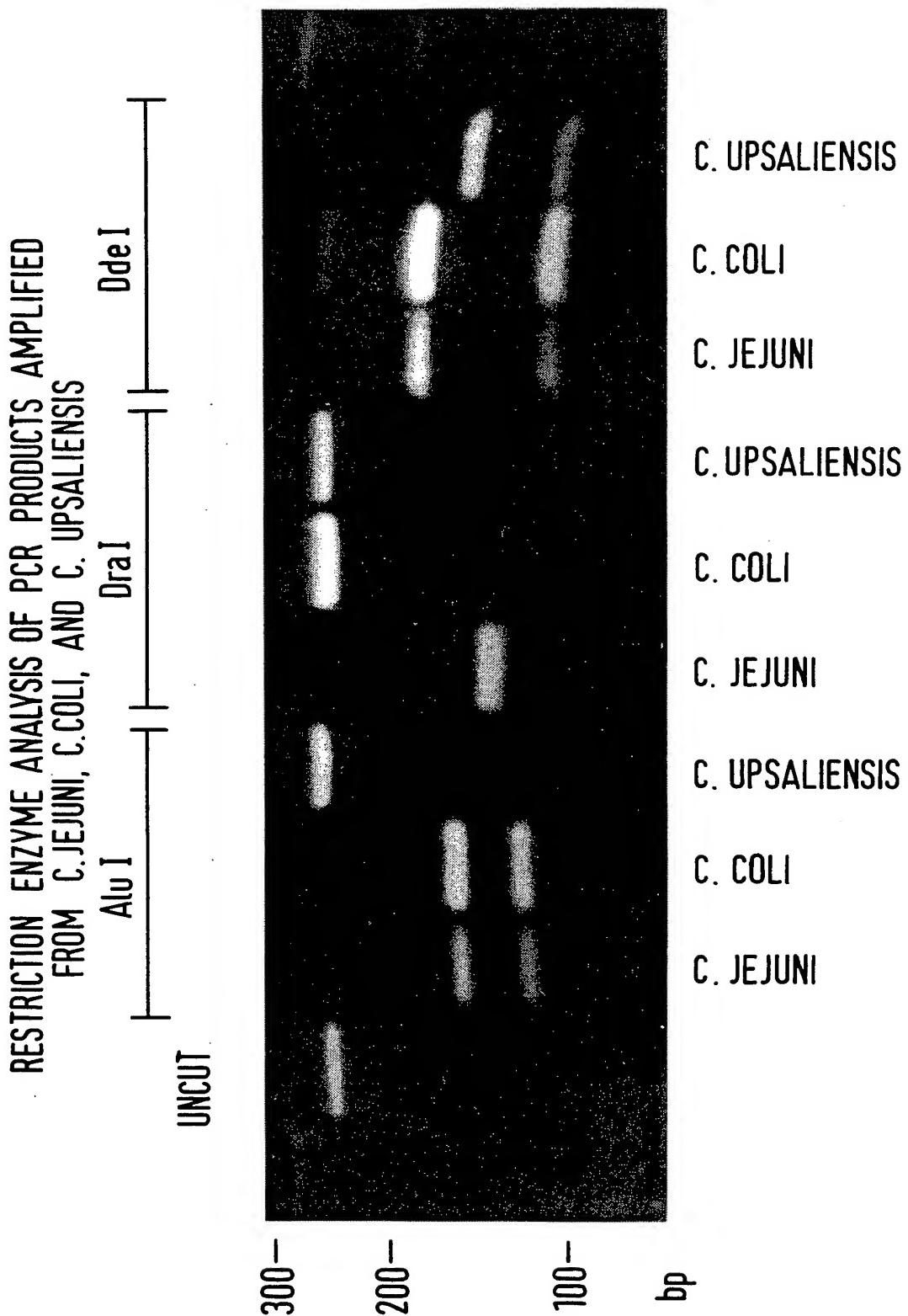
ctttcaggatttggcaacggatttatataaggcccggtttttaaaggtaggtatggc  
 -+-----+-----+-----+-----+-----+-----+  
 gaaagtccctaaccggttgcataatccaggccacaagaatttcactcataccg  
 L S C I W A T V L Y R S A C S \* S E Y G -

FIG. 2(II)

tgagtcaagattttaatggctgttgtt  
 -+-----+-----+-----+-----+-----+-----+ 1948  
 actcagttctaaaaattaccgacaacca  
 \* V K I F N G C W -

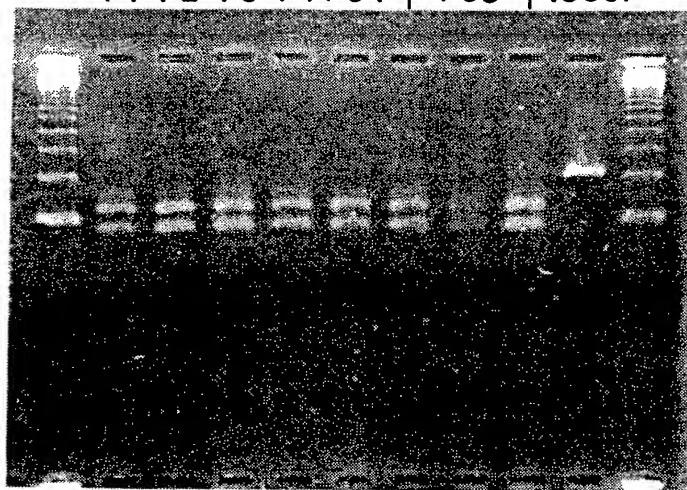
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FIG. 3



5 / 8

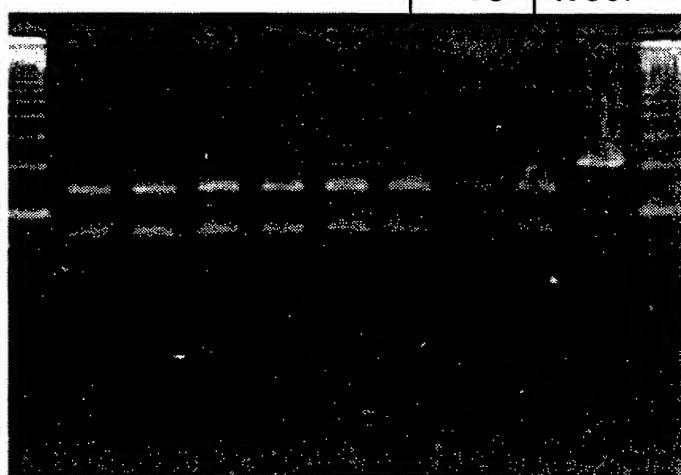
|    |    |    |    |     |       |        |       |
|----|----|----|----|-----|-------|--------|-------|
| P1 | P2 | P3 | P4 | P54 | P50   | C13997 | UNCUT |
|    |    |    |    |     | 13997 |        |       |



PENNER JEJUNI'S Alu I DIGEST

FIG. 4  
SIZE  
MARKERS  
ON ENDS

|     |        |       |    |     |     |       |
|-----|--------|-------|----|-----|-----|-------|
| P11 | C13997 | UNCUT |    |     |     |       |
| P1  | P2     | P3    | P4 | P54 | P50 | 13997 |



Dde I PENNER JEJUNI'S

FIG. 5

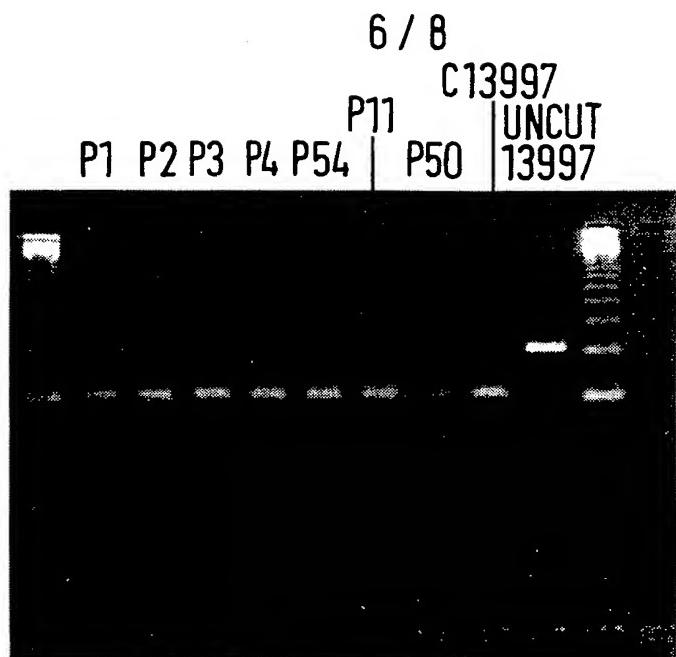
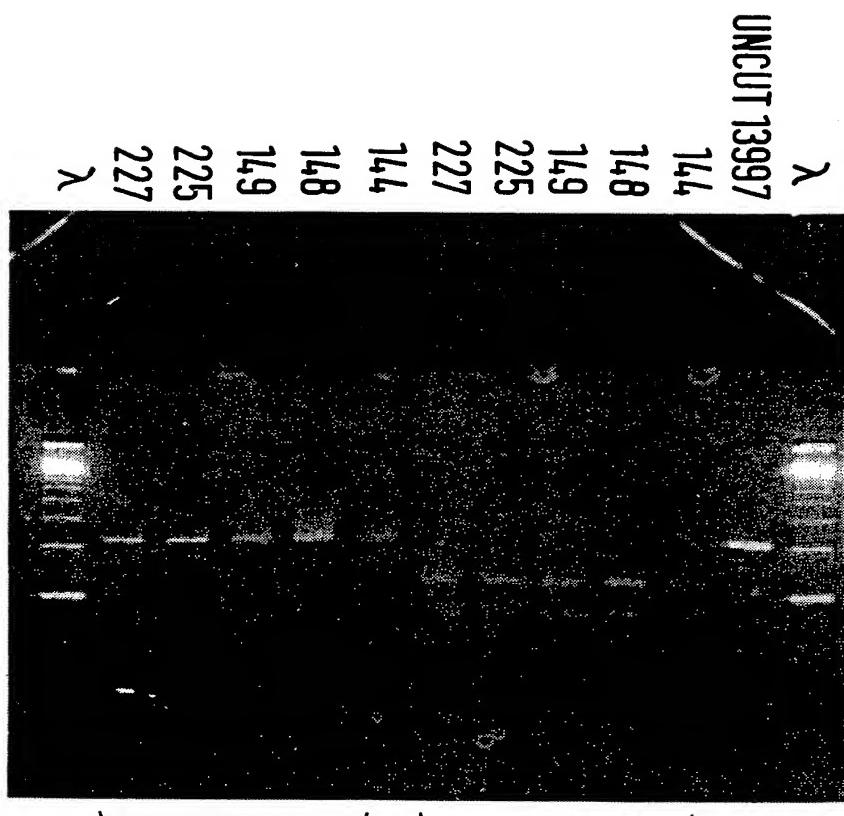


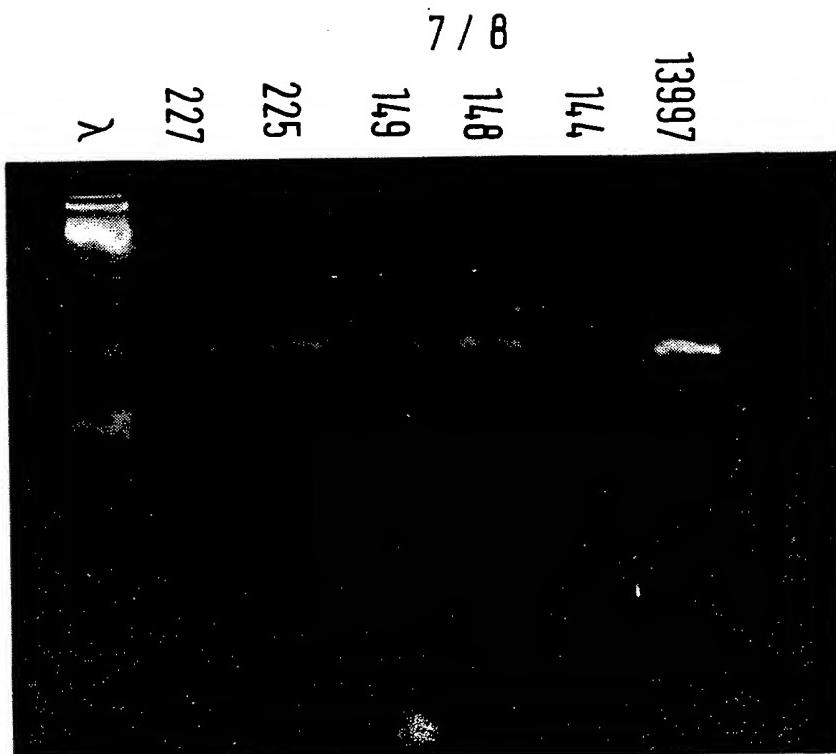
FIG. 6

Dra I PENNER JEJUNI'S



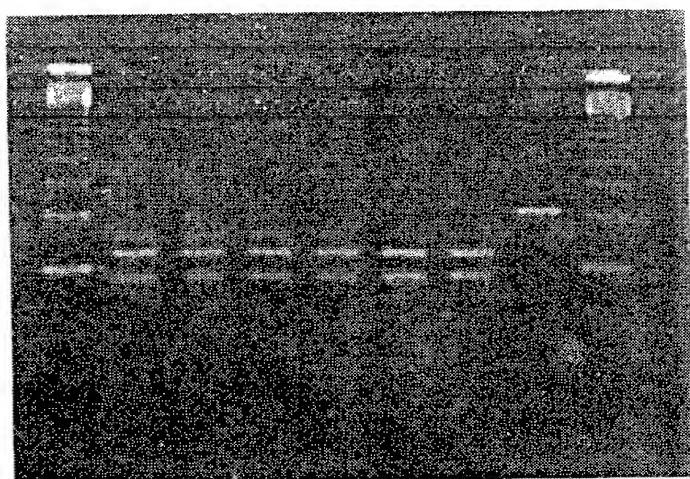
Alu I                  Dde I  
 UPSALIENSIS PCR's    Alu I    Dde I

FIG. 7



UPSALIENSIS PCR's Dra I CUT

P5 P24 P25 P59 L8 L2 UNDIG.  
COLI



Alu I - PENNER / LIOR COLI'S

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UNDIGESTED  
P5 P24 P25 P59 L8 L2 COLI

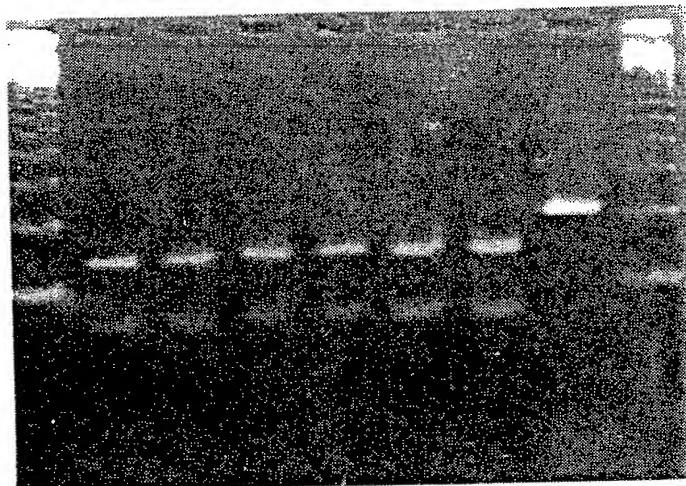


FIG. 10

C. Coli Dde I

P5 P24 P25 P59 L8 L21 UNDIG.  
COLI



FIG. 11

Dra I PENNER / LIOR COLI'S

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 94/01967

**A. CLASSIFICATION OF SUBJECT MATTER**  
**IPC 6 C12Q1/68**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
**IPC 6 C12Q**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| X          | J CLIN MICROBIOL 31 (6). 1993. 1531-1536.<br>NACHAMKIN I et al 'FLAGELLIN GENE TYPING<br>OF CAMPYLOBACTER -JEJUNI BY STRCTION<br>FRAGMENT LENGTH POLYMORPHISM ANALYSIS.'<br>see the whole document<br>--- | 10-15                 |
| X          | EP,A,0 350 392 (IRE-MEDGENIX S.A.) 10<br>January 1990<br>see claims 12,13; example 5<br>---   | 15                    |
| A          | LETTERS IN APPLIED MICROBIOLOGY 17 (5).<br>1993. 235-237.<br>Birkenhead D et al 'PCR for the detection<br>and typing of campylobacters.'<br>---   | 10-15                 |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

|   |   |
|---|---|
| Date of the actual completion of the international search<br><br><b>16 January 1995</b>   | Date of mailing of the international search report<br><br><b>10.02.95</b> |
| Name and mailing address of the ISA<br><br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Authorized officer<br><br><b>Molina Galan,-E</b>                          |

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 94/01967

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| A          | J BACTERIOL 175 (10). MAY, 1993.<br>3051-3057.<br>ALM R A et al 'DISTRIBUTION AND<br>POLYMORPHISM OF THE FLAGELLIN GENES FROM<br>ISOLATES OF MPYLOBACTER -COLI AND<br>CAMPYLOBACTER -JEJUNI.'<br>see the whole document<br>---           | 10-15                 |
| A          | APPLIED AND ENVIRONMENTAL MICROBIOLOGY,<br>vol.58, no.12, December 1992<br>pages 3804 - 3808<br>GIESENDORF ET AL. 'Rapid and sensitive<br>detection of C. spp. in chicken products<br>by using PCR'<br>cited in the application<br>----- |                       |

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

PCT/GB 94/01967

| Patent document cited in search report | Publication date | Patent family member(s) |         | Publication date |
|--|------------------|-------------------------|---------|------------------|
| EP-A-0350392                           | 10-01-90         | FR-A-                   | 2633941 | 12-01-90         |